

## THE SPIROKETALS CONTAINING A BENZYLOXYMETHYL MOIETY AT C8 POSITION SHOWED THE MOST POTENT APOPTOSIS-INDUCING ACTIVITY.

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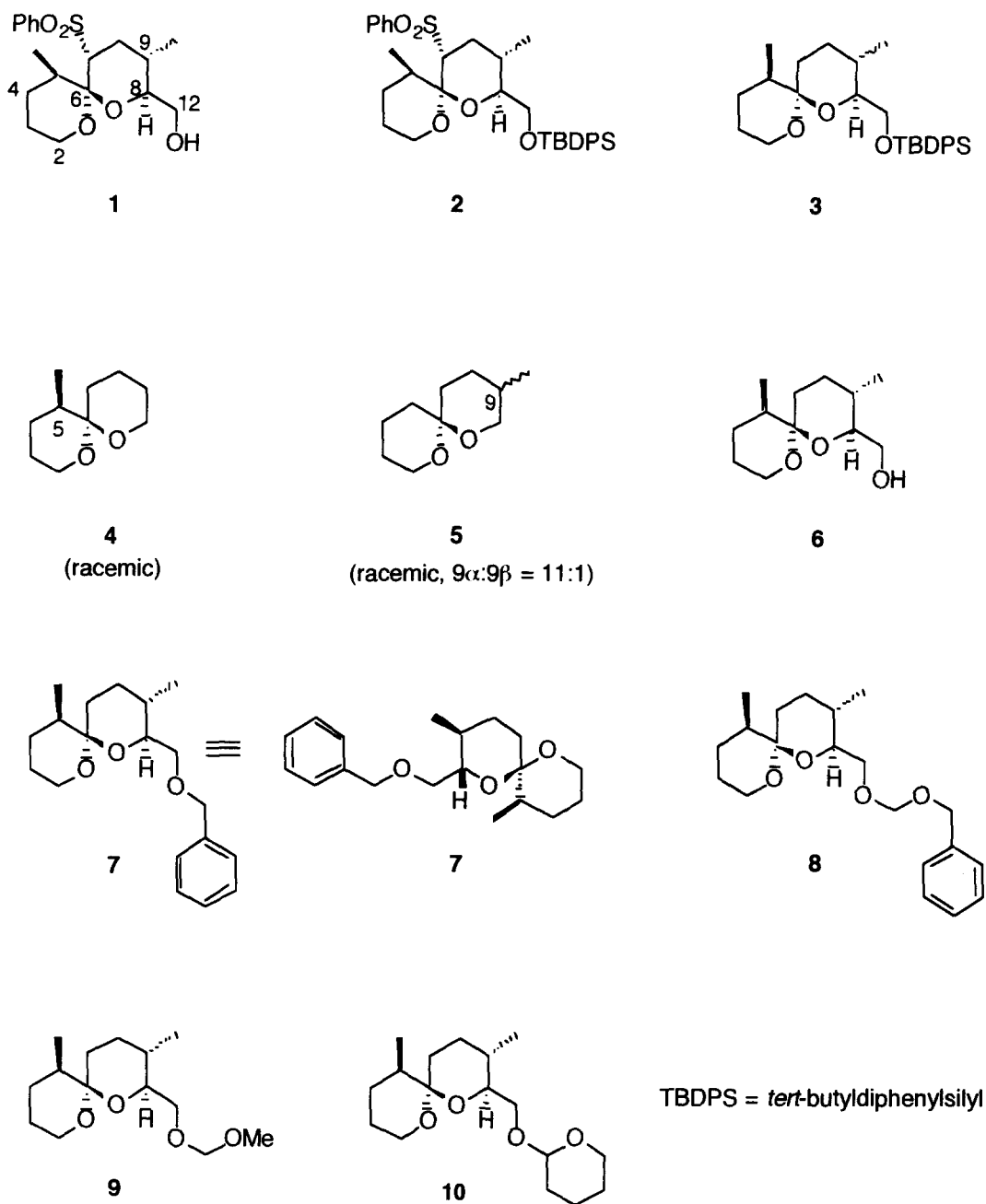
**Abstract:** The spiroketals containing a benzyloxy methyl moiety at the C8 position showed the most potent apoptosis-inducing activity, whereas its analogous compounds lacking any substituent at C8 or possessing ones other than the benzyloxy methyl moiety at C8 were all much less active. These results strongly suggest an important role of the benzyloxy methyl moiety linked to the C8 oxygen atom. © 1999 Elsevier Science Ltd. All rights reserved.

### Introduction

The previous evidence that apoptosis is induced by a Ser/Thr protein phosphatase inhibitor okadaic acid (OK) suggested that an inhibition of protein phosphorylation is involved in the apoptotic pathway (1, 2). To elucidate the possibility, we recently synthesized various derivatives of phosphatase inhibitors, tautomycin and thyriferyl 23-acetate, and examined their structure to function relationship (3, 4). We reported that the different moieties of tautomycin are separately involved in phosphatase inhibition and apoptosis induction. Throughout these experiments, it was found that all the tautomycin-related compounds with the apoptosis-inducing activity share a spiroketal structure. Furthermore, it should be noted that the spiroketal structure is present in the right wing of OK (5). These results motivated us to specify the minimum structure required for the apoptosis-inducing activity. In the present paper, we synthesized ten spiroketal-related compounds and measured their apoptosis-inducing activities.

### Results and Discussion

Ten spiroketal-related compounds were synthesized (Fig 1) and analyzed for their cytotoxicity to human T cell leukemia Jurkat cells under serum-deprived conditions (Table 1). We initially noticed that compound **3** shows a weak but significant apoptosis-inducing activity, whereas compounds **1** and **2** were less active. These results demonstrate that the addition of a substituent on the spiroketal structure attenuated the activity.

**Fig.1** Structures of various spiroketal compounds

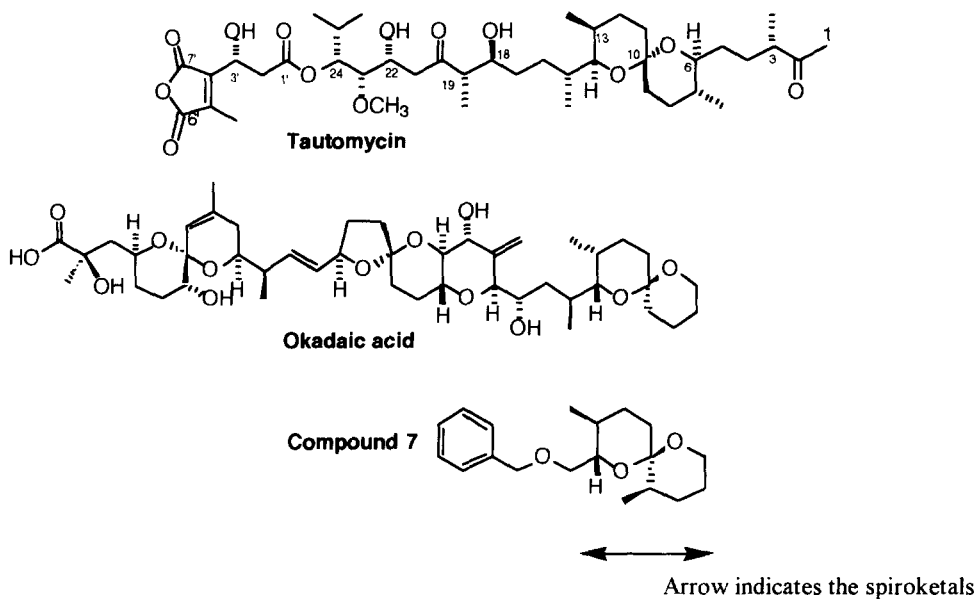
Simple spiroketal compounds **4** and **5** which do not have any substituent at C8 position did not show any cytotoxic effects on the cells. In contrast, compound **6** with the hydroxymethyl group at C8 position showed a weak but distinct cytotoxicity at a concentration of 100  $\mu\text{M}$ , demonstrating minimum size of C8 substituent required for the apoptosis-inducing activity. Therefore, compounds **4** and **5** were thought to be inactive presumably due to the absence of an appropriate substituent at C8 position. The most potent compounds **7** and **8** have a benzyloxymethyl moiety linked to the C8 position, suggesting the important role of a benzyloxymethyl moiety to fit appropriately to their putative target molecule(s). Concentrations of compounds **7** and **8** that cause 50 % lethality,  $\text{LC}_{50}$ , were 14  $\mu\text{M}$  and 32  $\mu\text{M}$ , respectively. In contrast to compounds **7** and **8**, compounds **9** and **10** had no or much less activities. Compound **10** is structurally similar to compounds **7** and **8**, but its activity was very weak probably because of the steric congestion at  $\alpha$  position of the C12 oxygen atom. The weak activity of compound **9** suggests the crucial role of the benzyloxymethyl moiety linked to the C8. Structure of compound **7** is similar to the C30-C38 spiroketal moiety in OK and dinophysistoxin-1 including absolute configuration (Fig. 2) (5). It is interesting to note that terminal spiroketal moieties of OK and tautomycin show apoptosis-inducing activity though they possess opposite absolute configuration at the spiroketal center. All the compounds examined seem to have low reactivity to amino acid residues. Thus, an irreversible binding of those compounds to the target protein(s) appears to be unlikely.

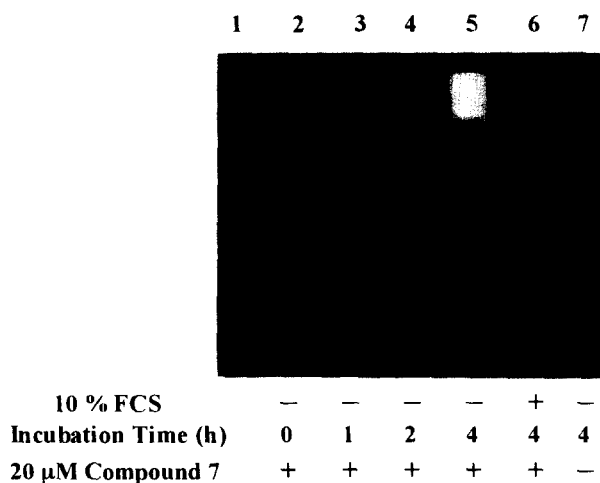
To elucidate a molecular basis for the cytotoxicity of the spiroketal compounds, the compound **7** with the most potent cytotoxic activity was used for further analyses. By a microscopic examination, cells began to die with typical apoptotic morphology such as nuclear fragmentation and chromatin condensation 4 h after incubation with compound **7** at a concentration of 20  $\mu\text{M}$  and almost all the cells died at 6 to 8 h. As shown in Fig. 3, DNA ladder appeared 1 h after incubation of the cells with compound **7**, increased for the subsequent 4 h, and then gradually decreased (data not shown). Since the appearance of the DNA fragmentation (6), which precedes the morphological change, is characteristic to apoptosis, the compound **7** was concluded to be a new potent apoptosis-inducer. The compound **8** with the second strongest activity also induced the DNA ladder formation (data not shown). There was a good correlation between the DNA ladder formation and the apoptosis-inducing activity of compounds **7** and **8**. We analyzed effects of these compounds on the phosphatase activity. None of the 10 compounds had any inhibitory activity towards PP1 or PP2A at a concentration of 100  $\mu\text{M}$  (data not shown). Compound **7**, which is free from the phosphatase inhibitory activity but with the most potent apoptosis-inducing activity could be used as a powerful tool for analysis of the mechanism for apoptosis.

**Table 1. Effects of spiroketal compounds on Jurkat cells.**

Compounds	Viable cells (%) $\pm$ SD	
	20 $\mu$ M	100 $\mu$ M
1	67.1 $\pm$ 3.4	39.0 $\pm$ 0.8
2	79.5 $\pm$ 1.7	68.4 $\pm$ 0.0
3	38.2 $\pm$ 4.8	43.0 $\pm$ 3.5
4	98.6 $\pm$ 1.6	87.4 $\pm$ 4.3
5	97.7 $\pm$ 0.0	84.6 $\pm$ 5.1
6	86.0 $\pm$ 1.4	43.8 $\pm$ 1.7
7	<b>1.7 <math>\pm</math> 2.5</b>	<b>0.0 <math>\pm</math> 0.0</b>
8	77.9 $\pm$ 14.9	<b>0.3 <math>\pm</math> 0.3</b>
9	89.1 $\pm$ 6.4	81.5 $\pm$ 4.3
10	84.1 $\pm$ 3.2	60.1 $\pm$ 9.6

Serum-deprived cells ( $5 \times 10^5$ /mL) were incubated with the indicated concentrations of spiroketal compounds for 24 h and cell viability were estimated by MTT assay. Under the control conditions without spiroketal compound, over 90 % of cells were viable and the cell numbers were almost constant. Values are average of at least three separate experiments.

**Fig. 2 Structures of tautomycin, okadaic acid, and compound 7 which contain commonly spiroketals.**



**Fig. 3 DNA fragmentation of Jurkat cells induced by Compound 7.** Cells were incubated with indicated conditions. DNA extracts ( $5 \times 10^5$  cells) were applied to 2.0% agarose gel containing 200 ng/mL ethidium bromide. Lane 1 is the DNA size marker ( $\phi$ x174, *Hae*III).

## Material and Method

**Cell line and Culture:** Human acute T lymphoblastic leukemia Jurkat cells were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 200  $\mu$ g/mL of streptomycin, and 50 U/mL of penicillin. Cells were cultured at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub>.

**MTT assay:** After washing three times with RPMI-1640 medium, cells were suspended at  $5 \times 10^5$ /mL with various concentrations of spiroketals compounds and plated in 96-well flat-bottom microtiter plates. Cells ( $5 \times 10^4$ /well) were cultured for 24 h, and then MTT was added at the final concentration of 0.5 mg/mL. After 4 h, 1 vol. of 0.04 M HCl/2-propanol was added, and the absorbance of the mixture was measured at 570 nm.

**Assay for DNA fragmentation:** The stimulated-cells ( $5 \times 10^6$ ) were suspended in 100 mL of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.5 % Triton X-100. The lysate was centrifuged at 8000 g for 20 min. The supernatant was incubated with 20  $\mu$ g RNase A at 37 °C for 1 h. Then 2  $\mu$ L of 20 mg/mL proteinase K was added and the mixture was incubated for 1 h. After the addition of 20  $\mu$ L of 5 M NaCl and 120  $\mu$ L of 2-propanol, the mixture was allowed to stand overnight at –20 °C. After centrifugation at 8000 g for 20 min, the pellet was suspended with 20  $\mu$ L TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA), mixed with 4  $\mu$ L of gel-loading buffer (40 % sucrose and 0.25 % Bromophenol Blue), and loaded on a 2 % agarose gel.

**Syntheses of compounds:** The synthesis of the compounds **1-4**, **6** and **7** were described in our previous papers (7, 8). Using an essentially same method as reported (8), the spiroketal **5** was prepared by treatment of THF with LDBB followed by addition of 4-methyl- $\delta$ -valerolactone (19%). **5** (major isomer): IR (NaCl) 2942, 1075  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ )  $\delta$  3.68-3.45 (3H, m), 3.24 (1H, t,  $J = 11.1$  Hz), 0.81 (3H, d,  $J = 6.6$  Hz); EI-HR-MS  $m/z$  170.1302 ( $\text{M}^+$ ,  $\text{C}_{10}\text{H}_{18}\text{O}_2$  requires 170.1293).

The compounds **8-10** were prepared from 8-substituted spiroketal **6** by standard procedures [**8**: BOMCl, *i*-Pr<sub>2</sub>NEt,  $\text{CH}_2\text{Cl}_2$  (78%). **9**: MOMCl, *i*-Pr<sub>2</sub>NEt,  $\text{CH}_2\text{Cl}_2$  (85%). **10**: 2,3-DHP, *p*-TsOH,  $\text{C}_6\text{H}_6$  (66%)]. **8**: IR (NaCl) 2931, 1042, 988  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ )  $\delta$  7.26-7.37 (5H, m), 4.90 (2H, s), 4.65 (2H, s), 3.55-3.74 (4H, m), 3.40 (1H, m), 0.90 (3H, d,  $J = 5.9$  Hz), 0.88 (3H, d,  $J = 5.9$  Hz); EI-HR-MS  $m/z$  334.4552 ( $\text{M}^+$ ,  $\text{C}_{17}\text{H}_{30}\text{O}_4$  requires 334.4558). **9**: IR (NaCl) 2933, 1042, 989  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ )  $\delta$  4.71 (1H, d,  $J = 5.9$  Hz), 4.68 (1H, d,  $J = 5.9$  Hz), 3.40-3.72 (4H, m), 3.39 (3H, s), 3.37 (1H, m), 0.90 (3H, d,  $J = 5.9$  Hz), 0.88 (3H, d,  $J = 5.9$  Hz); EI-HR-MS  $m/z$  258.3583 ( $\text{M}^+$ ,  $\text{C}_{17}\text{H}_{30}\text{O}_4$  requires 258.3580). **10**: IR (NaCl) 2933, 1031, 990  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ )  $\delta$  4.79 (1H, t,  $J = 3.0$ ), 4.74 (1H, t,  $J = 3.3$ ), 3.35-4.05 (7H, m), 0.89 (3H, d,  $J = 6.6$  Hz), 0.86 (3H, d,  $J = 6.6$  Hz); EI-HR-MS  $m/z$  298.2152 ( $\text{M}^+$ ,  $\text{C}_{17}\text{H}_{30}\text{O}_4$  requires 298.2144).

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